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Morphological and functional changes in guinea-pig neurons projecting to the ileal mucosa at early stages after inflammatory damage

Kulmira Nurgali[1], Zhengdong Qu[2], Billie Hunne[2], Michelle Thacker[2], Louise Pontell[2] and John B. Furness[2]

[1] *School of Biomedical and Health Sciences, Victoria University, Victoria 8001, Australia*
[2] *Department of Anatomy & Cell Biology, University of Melbourne, Parkville, Victoria 3010, Australia*

Non-technical summary Inflammation in the gut causes changes in neurons that control its movement and secretion. This leads to symptoms of pain and functional disorders that may persist long after the resolution of inflammation, which in humans manifests as the irritable bowel syndrome. In this study we demonstrate an association between hyperexcitability of neurons in the gut wall, damage to the nerve terminals in the mucosa and inflammation close to neurons and their terminals. These results increase our understanding of the triggering mechanisms that contribute to post-inflammatory gut dysfunctions.

Abstract In the present study the relationship between tissue damage and changed electrophysiological properties of Dogiel type II myenteric neurons within the first 24 hours after induction of inflammation with trinitrobenzene sulfonate (TNBS) in the guinea-pig ileum was investigated. Treatment with TNBS causes damage to the mucosa, inflammatory responses in the mucosa and enteric ganglia and changes in myenteric neuron properties. Thus we hypothesise that the physiological changes in the myenteric neurons could be due to damage to their mucosal processes or inflammation in the vicinity of cell bodies or the processes. We found an association between hyperexcitability of myenteric Dogiel type II neurons and damage to the mucosa and its innervation at 3 and 24 h, times when there was also an inflammatory reaction. The lack of hyperexcitability in neurons from control tissues in which axons projecting to the mucosa were severed suggests that inflammation may be an important contributing factor to the neuronal hyperexcitability at the acute stage of inflammation. Despite mucosal repair and re-innervation of the mucosa before 7 days after induction of inflammation, neuronal hyperexcitability persists. Although the mechanisms underlying neuronal hyperexcitability at the acute stage of inflammation might be different from those underlying long-term changes in the absence of active inflammation in the ganglia, the persistent changes in neuronal excitability may contribute to post-inflammatory gut dysfunctions.

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Corresponding author K. Nurgali: School of Biomedical and Health Sciences, Victoria University, PO Box 14428, Melbourne, Victoria 8001, Australia. Email: kulmira.nurgali@vu.edu.au

Abbreviations AH, afterhyperpolarizing; AHP, afterhyperpolarizing potential; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; RMP, resting membrane potential; TNBS, trinitrobenzene sulfonate.

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Introduction

Inflammation in both the small and large intestine results in hyperexcitability of specific classes of enteric neurons that long outlasts the period of inflammation in enteric ganglia (Linden *et al.* 2003; Lomax *et al.* 2005, 2007; Krauter *et al.* 2007*b*; Nurgali *et al.* 2007). There are also changes in neurotransmitter release and in synaptic transmission in the enteric nervous system (O'Hara *et al.* 2007; Krauter *et al.* 2007*a*; Hons *et al.* 2009; Nurgali *et al.* 2009). In published work, the electrophysiological properties of the enteric neurons were examined from 3 to 56 days after the induction of inflammation, when the acute inflammatory reaction has begun to subside or has substantially subsided. The consistent observation reported in these studies is that dramatic changes in excitability of Dogiel type II neurons occur following inflammation. These neurons have afterhyperpolarizing (AH) electrophysiological properties with prominent late afterhyperpolarizing potentials (AHPs) following their action potentials. They have large cell bodies and multiple processes, some of which project to the mucosa. The mucosal processes respond to physiologically relevant stimuli with action potentials that can be recorded from the cell bodies and output processes form synapses with other enteric neurons (Bertrand *et al.* 1997, 1998; Kunze *et al.* 1998, 2000). The responses of the neurons to mucosal and other stimuli and the connections that they make with other neurons imply that these are primary afferent neurons of enteric reflex pathways (Kirchgessner & Gershon, 1988; Furness, 2006).

The present study aims to investigate the relationship between tissue damage, associated axonal damage and changed electrophysiological properties of Dogiel type II myenteric neurons within the first 24 h after induction of inflammation with trinitrobenzene sulfonate (TNBS) in the guinea-pig ileum. Treatment with TNBS causes damage to the mucosa and initiates an inflammatory response in the myenteric plexus that is associated with changes in myenteric neuron properties (Linden *et al.* 2005).

It has been suggested that long-lasting hyperexcitability of enteric neurons following intestinal inflammation, as well as synaptic facilitation in enteric circuits, might contribute to symptoms of pain and disorders of motility that persist long after the resolution of inflammation, which is manifest in patients as the irritable bowel syndrome (IBS), to altered gut function during periods of remission from inflammatory bowel disease (IBD), and to functional disorders of the gastrointestinal tract (Camilleri, 2004; De Giorgio *et al.* 2004; Mawe *et al.* 2009). We hypothesize that preventing effects on neurons at the acute stage of inflammation could circumvent the triggering mechanisms of neuronal hyperexcitability, and, therefore, the development of post-inflammatory gut dysfunctions. To be able to do this, the early time course of change needs to be known.

Methods

All experiments were performed on guinea-pigs (180–250 g) of either sex from the inbred Hartley strain colony of the Department of Anatomy and Cell Biology at the University of Melbourne. All procedures were conducted according to the Code of Practice of the National Health and Medical Research Council of Australia and were approved by the University of Melbourne Animal Experimentation Ethics Committee, and comply with the policies and regulations of *The Journal of Physiology* (Drummond 2009). All animals were maintained in a controlled environment at 21°C on a 12:12 h light–dark cycle with free access to food and water. At the time of taking tissue, animals were stunned by a blow to the head and killed by cutting the carotid arteries and severing the spinal cord.

Induction of inflammation

Guinea-pigs were anaesthetized with a mixture of xylazine (20 mg kg^{-1}) and ketamine hydrochloride (100 mg kg^{-1}, Troy Laboratories, Australia), given intramuscularly. The abdomen was opened by a 1.5 cm incision in the mid-line and the distal part of the ileum was exteriorized. 2,4,6-Trinitrobenzenesulfonic acid (TNBS; Wako Industries, Nagoya, Japan), 30 mg kg^{-1} in 1 ml of 30% ethanol, was injected into the lumen of the ileum, approximately 8 cm proximal to the ileoceacal junction, through a 30 gauge needle over the course of 1 min. The intestine was temporarily occluded just distal to the injection site during the injection and for a further minute following injection. A fine silk ligature (5.0) was tied loosely around a nearby blood vessel to mark the injection site for later location. The intestine was then returned to the abdominal cavity, the abdominal wall and peritoneum were closed with sutures, and the skin was closed with stainless steel staples. The guinea-pigs were housed individually and monitored during recovery from anaesthesia (1–2 h) and then housed together, with free access to food and water. The guinea-pigs showed no signs of stress, exhibited apparently normal exploratory behaviour, and ate soon after they awoke from anaesthesia. Animals were taken at 3, 24 h and 7 days after TNBS injection. Segments of inflamed ileum were removed for electrophysiological and immunohistochemical studies and histological assessment of the degree of inflammation. Animals were weighed prior to administration of TNBS and daily following surgery.

Control data were obtained from guinea-pigs of the same colony that were in the same age and weight range, but which were not subjected to surgery. In addition, changes were investigated in tissues from sham-operated animals. These animals underwent identical surgery, substituting the injection of physiological saline into the lumen of the ileum instead of TNBS.

Tissue preparation for electrophysiology

Segments of ileum, 2–3 cm long, were taken from the inflamed regions in TNBS-treated animals 3 and 24 h after TNBS injection, from sham-operated guinea-pigs at the same time points and from control (untreated) animals, 8–10 cm proximal to the ileocaecal junction. The segments were placed in physiological saline (composition in mM: NaCl 118, KCl 4.8, $NaHCO_3$ 25, NaH_2PO_4 1.0, $MgSO_4$ 1.2, glucose 11.1, $CaCl_2$ 2.5; equilibrated with 95% O_2–5% CO_2) and initially kept at room temperature. The solution contained 3 μM nicardipine and 1 μM hyoscine (both from Sigma-Aldrich, Sydney, Australia) to inhibit muscle movement. The mucosa, submucosa and circular smooth muscle were carefully removed to expose the myenteric plexus. The preparation was pinned to the Sylgard elastomer (Dow Corning) base of a recording dish (volume 1 ml), which was placed on the stage of an inverted microscope and continuously superfused (4 ml min^{-1}) with physiological saline that had been preheated to yield a bath temperature of 34–35°C. The tissue was equilibrated with perfusate for 1–2 h before recording commenced.

Electrophysiological recordings

Neurons were impaled with conventional borosilicate glass microelectrodes filled with 1% biocytin (Sigma-Aldrich, Australia) in 1 M KCl. Electrode resistances were 100–170 MΩ. Recordings were made using an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA). Signals were digitized at 1–10 kHz, using a Digidata 1322A interface (Molecular Devices) and stored using PC-based data acquisition software (Axoscope 8.2, Molecular Devices). Measurements of electrophysiological properties were made after allowing the impalements to stabilize for at least 15 min without applying intracellular holding current. At this time the ability of the cell to fire an action potential was assessed. Action potentials were evoked both by extracellular stimulation to induce antidromically activated action potentials and by injection of brief intracellular current pulses. Only cells that were able to fire an action potential, had resting membrane potentials (RMPs) more negative than −40 mV, and were adequately filled with dye to reveal their morphology were included in the electrophysiological analysis. The properties of the action potentials and late AHPs in all groups were recorded at −60 mV, which was achieved by injection of holding current when necessary.

Small intracellular hyperpolarizing current pulses (duration 500 ms, intensity 30 pA, yielding voltage shifts of 5–10 mV) were used to determine input resistance (R_{in}) and cell capacitance (C_{in}). Excitability was assessed by injecting 0.5–2 s depolarizing current pulses, at an intensity of 20–300 pA at 20 s intervals, through the recording electrode while a holding current was used to maintain the resting membrane potential at −60 mV between pulses.

Electrical stimuli were applied to interganglionic connectives using a fine tungsten stimulating electrode (10–50 μm tip diameter), insulated except at the tip. The stimulating electrode was positioned at the end of the ganglion, circumferential to the recording electrode, so that the inputs from most processes including circumferential, ascending and descending inputs were stimulated. Stimuli were delivered via an ISO-Flex stimulator controlled by a Master-8 programmable pulse generator (both from AMPI, Jerusalem, Israel). Fast excitatory postsynaptic potentials (EPSPs) were evoked by extracellular pulses of 0.1 ms duration and 0.3–0.5 mA intensity at 10 s intervals while the membrane potential was held at −90 mV. Slow EPSPs were evoked by 20 Hz trains of 0.1 ms pulses at an intensity of 0.08–0.5 mA for 1 s. To determine input resistance during slow postsynaptic events, small hyperpolarizing current pulses (duration 100 ms, intensity 20–50 pA) were injected. All parameters were determined using in-house analysis routines written in Igor Pro 4.0 analysis software (WaveMetrics, Inc., Lake Oswego, OR, USA).

Neuron identification

Biocytin was passed from the recording electrodes into the neurons during impalement. Once a neuron in a ganglion had been injected with biocytin, a diagram of the positions of the ganglion and of the impaled neuron was prepared so that the neuron could be later identified under the microscope. If further recordings were taken, the electrode was moved to a fresh ganglion to avoid ambiguity of cell identity. At the end of each experiment, the tissue was fixed overnight in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.0), cleared in three changes, 10 min each, of dimethylsulphoxide (DMSO), and washed in phosphate-buffered saline (PBS, 3 × 10 min). Fixed tissue was stored at 4°C in PBS containing sodium azide (0.1%) overnight. The tissues were then washed (3 × 10 min) in PBS prior to incubation with streptavidin coupled to Texas red (GE Healthcare (Amersham), Rydalmere, NSW, Australia), 1:400, for 1 h at room temperature. Preparations were then washed (3 × 10 min) in PBS and mounted on glass slides using buffered glycerol (pH 8.4).

To analyse the morphologies and projections of the impaled neurons, preparations in which impaled nerve cells had been identified were removed from the slides and washed in PBS, prior to conversion of the

Table 1. Primary antibodies and labels used in this study

Tissue antigen	Host species	Dilution	Source or reference
β-Tubulin III (TUJ1)	Mouse	1:2000	Covance (Emeryville, CA, USA)
Calbindin	Rabbit	1:1000	SWant (Bellinzona, Switzerland)
NeuN	Mouse	1:200	Chemicon (Temecula, CA, USA)
Hu, ELAV protein	Human	1:2000	Fairman *et al.* (1995)

Table 2. Secondary antibodies used in this study

Antibody and label	Dilution	Source
Donkey anti-rabbit IgG, Alexa 488	1:100	GE Healthcare (Amersham), Rydalmere, NSW, Australia
Donkey anti-mouse IgG, Alexa 594	1:200	Invitrogen, Mulgrave, Australia
Donkey anti-human IgG, Texas red	1:100	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Goat anti-mouse IgG, Alexa 488	1:200	Invitrogen, Mulgrave, Australia
Donkey anti-rabbit IgG, horseradish peroxidase	1:1200	GE Healthcare (Amersham), Rydalmere, NSW, Australia

streptavidin, bound to the biocytin, to a permanent deposit (Clerc *et al.* 1998). This was achieved using goat anti-streptavidin antiserum coupled to biotin (Vector Laboratories, Burlingame, CA, USA), diluted at 1:50 and incubated overnight at 4°C. The biotin was in turn localized using an avidin–biotin–horseradish peroxides kit (Vectastain, Vector Laboratories). The horseradish peroxidase was reacted with diaminobenzidine and hydrogen peroxide to yield a permanent deposit. Cell shapes, positions and projections were evaluated on an Olympus BH microscope under positive-low phase contrast optics, and drawn with the aid of a camera lucida drawing tube at ×400 or ×1000 magnification.

Immunohistochemistry

For immunohistochemical studies, segments of ileum were taken from control guinea-pigs, from animals with TNBS-induced inflammation at 3, 24 h and 7 days after TNBS injection and from sham-operated animals at the same time points after the injection of physiological saline.

For tissue to be examined in wholemounts, segments of the ileum were placed in PBS (0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) containing nicardipine (1 μM), cleaned of contents, opened and pinned tautly, mucosa side down, onto balsa board. They were then fixed overnight at 4°C in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.0). The next day, tissue was cleared of fixative in 3 × 10 min washes in DMSO, followed by 3 × 10 min washes in PBS. For tissue to be examined in section, segments of the ileum were removed, pinned to balsa board mucosa side up without stretching and fixed overnight at 4°C. Tissue was cleared in DMSO as described above and placed in PBS–sucrose–azide (PBS containing 0.1% sodium azide and 30% sucrose as a cryoprotectant) and stored at 4°C overnight. The following day, small segments of tissue were transferred to a mixture of PBS–sucrose–azide and OCT compound (Tissue Tek, Elkhart, IN, USA) at a ratio of 50:50 for a further 24 h before being embedded in 100% OCT. Transverse cryostat sections of 30 μm thickness were cut and collected on gelatinized (1%) microscope slides and left to dry for 1 h at room temperature prior to immunostaining. Nerve fibre bundles were labelled with anti-β-tubulin III antibody followed by double-staining with anti-calbindin antibody specific to Dogiel type II neurons (Table 1). The total number of neurons in the myenteric plexus was quantified using anti-Hu immunoreactivity and the number of Dogiel type II neurons was quantified using anti-cytoplasmic NeuN immunoreactivity at 24 h and 7 days after TNBS injection (Table 1). Tissues were incubated in primary antibodies overnight at 4°C. The tissues were then washed (3 × 10 min) in PBS prior to incubation with the secondary antibodies (Table 2) for 1 h at room temperature. Tissues were washed (3 × 10 min) in PBS before they were mounted in Dako fluorescence mounting medium (Dako North America Inc., Carpinteria, CA, USA). Preparations were also analysed by confocal microscopy on a Zeiss Pascal confocal laser scanning system. Fluorophores were visualized using a 488 nm excitation filter and 522/535 nm emission filter for Alexa 488 or FITC, and 568 nm excitation and 605/632 nm emission filter for Alexa 594. Z-series images and single 1024 × 1024 pixel images (optical sections of 0.5 μm nominal thicknesses) were captured. The images were further processed using laser scanning microscope image browser, Image J, Corel Photo Paint and Corel Draw software programs.

Statistics

Data are presented as mean ± S.E.M. Statistical differences were determined by Student's *t* test (paired and unpaired), chi-square test and one-way ANOVA with Tukey–Kramer *post hoc* test for multiple group comparisons. Differences were considered statistically significant at $P < 0.05$.

Results

TNBS caused inflammation of a 5–8 cm length of the ileum as has been previously reported (Nurgali *et al.* 2007; Pontell *et al.* 2009). Segments of the ileum from guinea-pigs with TNBS-induced inflammation, sham operation and control animals were taken for electrophysiological, histological and immunohistochemical studies.

Electrophysiological properties of myenteric neurons at early stages of intestinal inflammation

Segments of the ileum were taken from 12 guinea-pigs with TNBS-induced ileitis at 3 h and 21 guinea-pigs at 24 h after TNBS injection, seven sham-operated guinea-pigs at 3 h and eight guinea-pigs at 24 h after physiological saline injection and 12 control (untreated) animals.

All neurons included in analyses were characterized electrophysiologically and adequately labelled by intracellular injection of biocytin during recording. AH neurons, the focus of this study, were characterized by the presence of a late afterhyperpolarizing potential (AHP) following one or more action potentials and an inflection on the repolarizing phase of the action potential. Morphologically, AH neurons were identified as multiaxonal Dogiel type II neurons that had large round or oval cell bodies. Few AH neurons had Dogiel type I morphology (3/22 in the 24 h TNBS group, none in the sham-operated or control groups) with a single axon and irregular lamellar dendrites (Furness, 2006).

Excitability of Dogiel type II neurons. All Dogiel type II neurons recorded from the inflamed ileum at 3 h (18/18) and 24 h (19/19) post-TNBS injection were significantly hyperexcitable compared to Dogiel type II neurons from the control ileum ($n = 9$) (Figs 1 and 2).

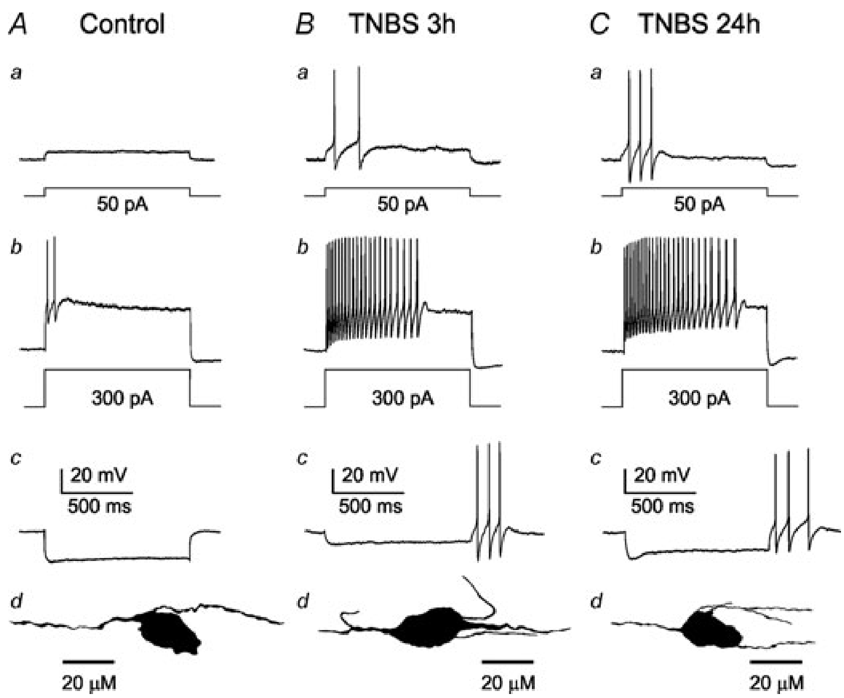

Figure 1. Recordings from Dogiel type II (DII) neurons from the control and inflamed ileum at different time points after induction of inflammation by TNBS
In each set of panels, *a* is the response to a 50 pA, 1s intracellular depolarizing current pulse; *b* is the response to 300 pA, 1s depolarization; *c* shows anodal break action potentials induced by intracellular hyperpolarization; *d* shows the shape of the neuron that was revealed by injecting dye during recording and drawn with the aid of a camera lucida. *A*, recordings from a DII neuron from the control ileum: *a*, 50 pA intracellular depolarizing current pulse did not induce action potential firing; *b*, two action potentials were fired in response to 300 pA; *c*, no anodal break action potentials were induced in this neuron. *B*, recordings from a DII neuron from the inflamed ileum taken 3 h after TNBS injection: *a*, 50 pA depolarizing current induced two action potentials; *b*, a train of action potentials was fired in response to 300 pA; *c*, a burst of anodal break action potentials occurred after intracellular hyperpolarization *C*, recordings from a DII neuron from the inflamed ileum taken 24 h after TNBS injection were similar to 3 h post TNBS: *a*, three action potentials were induced by 50 pA depolarization; *b*, a train of action potentials was fired in response to 300 pA; *c*, a burst of anodal break action potentials was induced by intracellular hyperpolarization.

The threshold for depolarizing current pulses to evoke an action potential was significantly lower at 3 and 24 h after TNBS injection than for neurons from the control ileum ($P < 0.05$) and the number of action potentials fired in response to the maximum (300 pA) depolarizing current pulse was significantly greater compared to Dogiel type II neurons from the control ileum ($P < 0.05$, Fig. 2). Most of the Dogiel type II neurons from sham-operated guinea-pigs had electrophysiological parameters similar to neurons from the control ileum. However 3/9 neurons at 3 h after sham operation were hyperexcitable. Thus, in sham-operated animals at 3 h after the surgery the averages of both the threshold and the number of action potentials in response to 300 pA depolarization were not different from neurons at 3 h post-TNBS injection (Fig. 2). At 24 h after the sham operation these parameters returned to control levels and were significantly different from those in the 24 h TNBS group ($P < 0.05$, Fig. 2).

The presence of anodal break action potentials induced by hyperpolarizing current pulses is another measure of neuronal hyperexcitability. Neurons from the inflamed ileum showed prominent anodal break action potentials with burst firing at 3 and 24 h after TNBS injection (Fig. 1) often in response to a very small amplitude hyperpolarizing current pulse (Fig. 1*Bc*). The proportions of Dogiel type II neurons that exhibited anodal break action potentials were 53% (8/15) at 3 h and 44% (8/18) at 24 h after TNBS injection. These were significantly greater compared to Dogiel type II neurons from the control ileum (8%, 1/13) ($P < 0.05$, chi-square test). Anodal break action potentials also occurred in 3/9 neurons from the 3 h sham operated group, but none of the neurons at 24 h after sham operation had anodal break action potentials. Spontaneous action potentials were recorded in two neurons (one at 3 h and one at 24 h) after TNBS injection. No spontaneous action potentials were observed in neurons from sham or control animals.

Passive and active membrane parameters of Dogiel type II neurons. There were no changes in the amplitudes of the action potentials at any time point after induction of inflammation in the ileum or after sham operations, compared to control (Fig. 3*C*). However, the durations of the action potentials, measured as the width at the action potential at half amplitude, were significantly increased at 3 h after TNBS injection compared to the control group ($P < 0.05$, Fig. 3*D*). The increase in the duration of the action potentials was also observed in the 24 h sham operated group. This was the only action potential parameter that was significantly different at 24 h after sham operation compared to controls. The amplitude of the late AHP following an action potential was significantly reduced in neurons from the inflamed ileum at 3 and 24 h after TNBS injection compared to the control group ($P < 0.05$, Fig. 3*E*). No significant differences in AHP duration were found between groups (Fig. 3*F*). The cell input resistance was greater at 3 h after TNBS injection

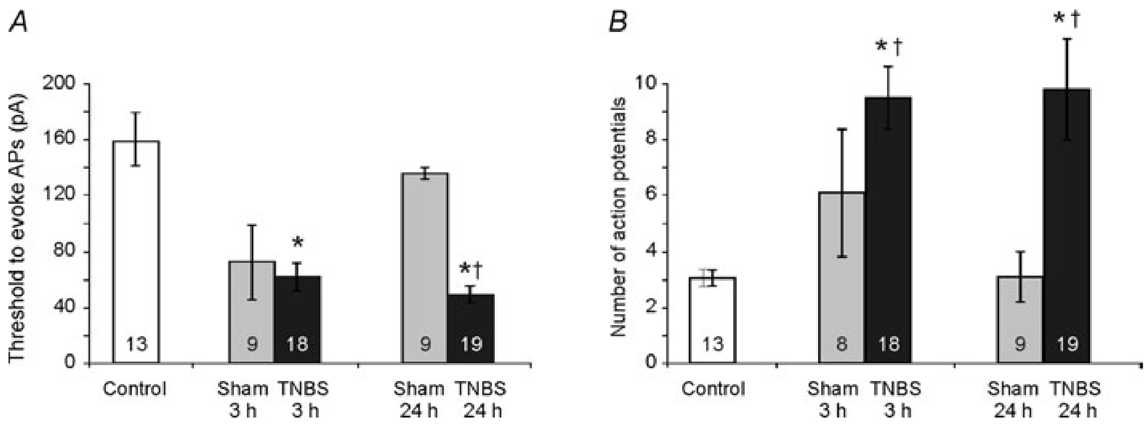

Figure 2. Neuronal excitability measured as a threshold to evoke action potentials in response to intracellular depolarizing current injection (*A*) and as a response to maximum depolarization of 300 pA (*B*)

Data were obtained from Dogiel type II (DII) neurons from the control ileum, DII neurons from sham operated guinea-pigs taken at 3 and 24 h after injection of saline into the ileum and DII neurons from inflamed ileum taken at 3 and 24 h after injection of TNBS into the ileum. *A*, thresholds for action potential generation in DII neurons from the inflamed ileum were significantly lower than from control at all time points after TNBS injection ($P < 0.05$) and significantly lower than from sham operated guinea-pigs at 24 h ($P < 0.05$). *B*, the number of action potentials elicited by 300 pA (maximum depolarization used in this study) pulses was significantly greater in DII neurons from the inflamed ileum taken at 3 and 24 h after TNBS injection compared to control ileum ($P < 0.05$). *Significantly different from DII neurons of the control group ($P < 0.05$). †Significantly different from DII neurons of the 24 h sham operated group ($P < 0.05$).

and at 3 h after sham operation compared to control guinea-pigs (Fig. 4*A*). There were no differences found in the resting membrane potential and cell capacitance between any groups (Fig. 4*B* and *C*).

AH neurons with fast EPSPs. All neurons in this study were tested for the presence of fast EPSPs, with the membrane potential offset to –90 mV. Fast EPSPs were recorded from 4/22 neurons with late AHPs at 24 h and

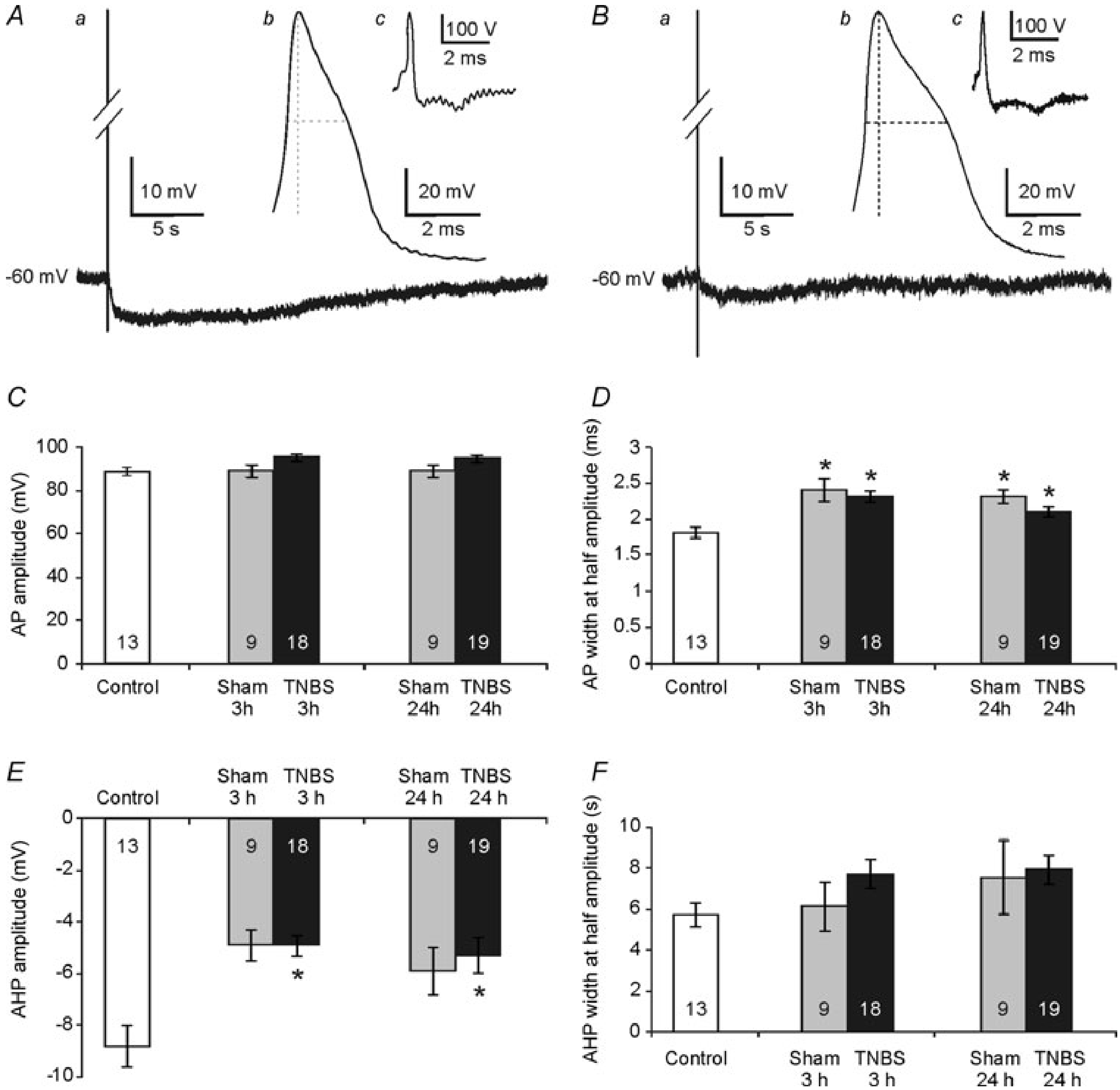

Figure 3. Properties of the action potential and the late afterhyperpolarization (AHP) in myenteric Dogiel type II (DII) neurons from the control, sham operated and inflamed ileum

A, the late AHP (*a*), action potential shape (*b*) and the first derivative (d*V*/d*t*) of the action potential (*c*) in a DII neuron from the control ileum. *B*, recording from a DII neuron from the inflamed ileum taken 24 h after TNBS injection: the amplitude of the late AHP is smaller (*a*) and the duration of the action potential is longer (*b*) in this neuron compared to control (*A*). The first derivative (d*V*/d*t*) of the action potential (*Bc*) shows that there is no changes in the rising phase of the action potential. *C*, action potential amplitudes of DII neurons were not significantly affected by inflammation or sham operation at any time points. *D*, action potential durations measured as width at half-amplitude were significantly longer in both DII neurons from inflamed ileum taken at 3 and 24 h after injection of TNBS into the ileum and sham operated guinea-pigs taken at 3 and 24 h after injection of saline into the ileum compared to neurons from control guinea-pigs ($P < 0.05$). *E*, late AHP amplitudes were significantly inhibited in the DII neurons from the inflamed ileum at 3 and 24 h after TNBS injection and sham operation compared to control groups ($P < 0.05$). *F*, no significant differences were found in the duration of the late AHP between all groups. *Significantly different from DII neurons of the control group ($P < 0.05$).

1/18 AH neuron at 3 h after TNBS injection. Analysis of their morphology revealed that two of these neurons had Dogiel type II morphology (one from the 3 h group and one from the 24 h group) and three of these neurons (all from the 24 h group) had Dogiel type I morphology (Fig. 5). None of the AH neurons from the sham operated groups at any time points had fast EPSPs.

Evidence of axonal damage following induction of inflammation

Innervation of villi. A monoclonal antibody against neuronal class III β-tubulin (TUJ1) was used in this study. Immunoreactivity for β-tubulin III was seen in the neuronal cell bodies and processes innervating smooth muscles and mucosa, but was not observed in glial cells. In sections from the control ileum ($n = 3/3$), β-tubulin immunoreactive fibres were distributed throughout the villous cores and formed a dense network at the base of the villi (Fig. 6*A*). In sections from the inflamed ileum at 3 and 24 h after TNBS injection ($n = 3$ for each time point), which contained either damaged villous profiles or no villi at all, β-tubulin immunoreactive fibres were fragmented, irregularly distributed or retracted from the epithelial surface (Fig. 6*C* and *E*). At 7 days after the induction of inflammation, dense innervation of the villi and nerve fibres in the thickened muscles were labelled by β-tubulin (Fig. 6*G*). In the tissues from 7 day sham operated animals, the distribution of β-tubulin immunoreactive fibres ($n = 3/3$) was similar to tissues from control guinea-pigs (Fig. 6*F*).

Histological examination of sections from sham operated guinea-pigs was performed at 3, 24 h and 7 days after saline injection ($n = 3$ for each time point) and compared to the sections from control ($n = 2$) and inflamed guinea-pigs ($n = 3$ at each time point). In the shams at 3 h the villi were intact. This contrasts to the TNBS preparations at 3 h, in which the villi were almost entirely gone and the epithelium was almost completely missing from the surfaces between the villi (Fig. 7). There was a low level accumulation of inflammatory cells (lymphocytes, eosinophils and neutrophils) at the tips of villi at 3 h in the shams, but the villus epithelium was intact (Fig. 7*C*). There were no changes detected in or around the myenteric ganglia or in the external muscle in the shams.

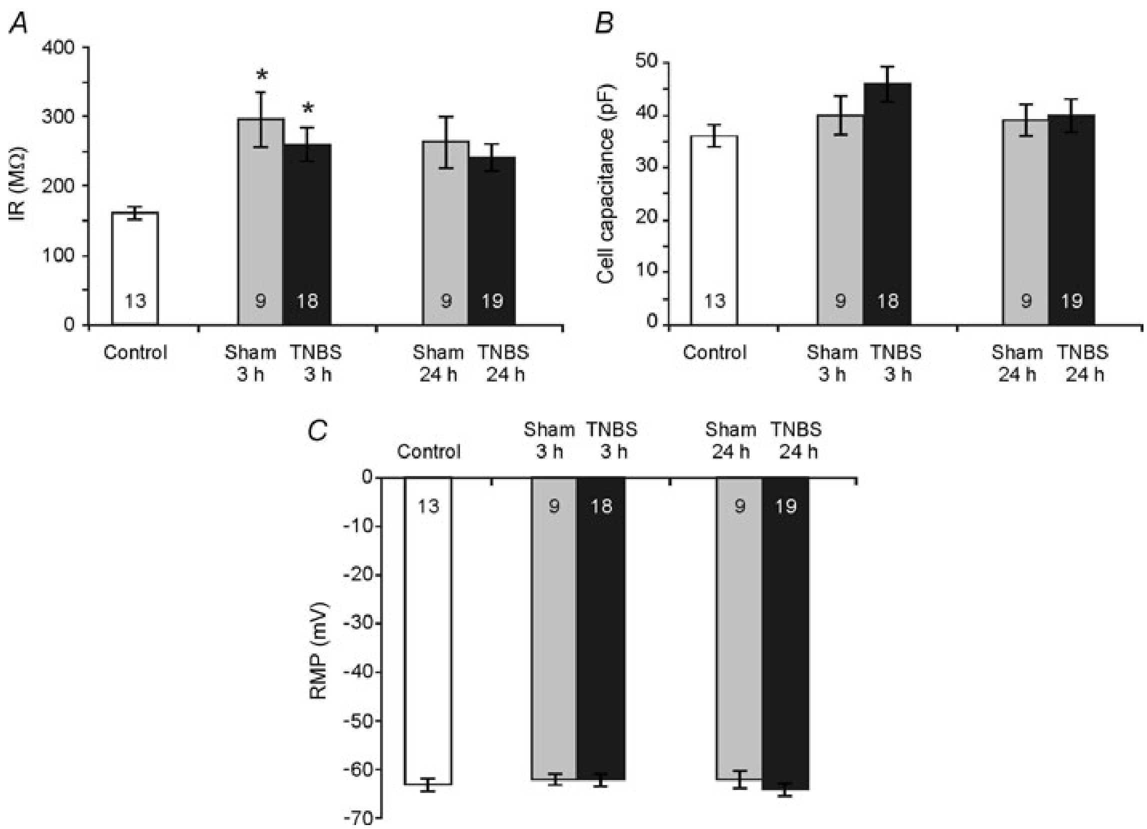

Figure 4. Comparisons of the input resistance (IR), cell capacitance and the resting membrane potential (RMP) of Dogiel type II (DII) neurons from the control, sham operated and inflamed ileum

A, input resistance was significantly higher in DII neurons from 3 h TNBS and 3 h sham operated groups compared to control guinea-pigs. No significant changes in the cell capacitance (*B*) and RMP (*C*) between groups were found. *Significantly different from DII neurons of the control group ($P < 0.05$).

No differences from normal were apparent in the tissues from shams at 24 h after the surgery. In contrast, the epithelium has not returned to normal after 24 h following TNBS treatment.

In this study, we used double staining with an anti-calbindin antibody specific for Dogiel type II neurons in the guinea-pig enteric nervous system (Quinson *et al.* 2001) which showed that β-tubulin immunoreactive nerve bundles also contained calbindin-positive fibres in sections from both control and inflamed ileum (Fig. 8). Cell bodies of Dogiel type II neurons in the myenteric plexus were also labelled by both anti-β-tubulin and anti-calbindin antibodies (Fig. 8).

Increase in number of nerve fibres following induction of inflammation. Transverse sections were taken at the middle level of villi in tissues from the control ($n = 3$) and inflamed ileum at 7 days post-TNBS injection ($n = 4$). Mid-villi sections were not possible to cut at early stages of inflammation as the villi were significantly damaged and restored to their full heights only at day 7 after TNBS injection. Analyses of mid-villi sections showed increased number of β-tubulin immunoreactive nerve fibre bundles innervating the mucosa. The diameter of fibres in the sections from the inflamed ileum appeared to be smaller compared to sections from control guinea-pigs (Fig. 9).

To investigate if the increase in the number of fibre bundles in the sections from the inflamed ileum was due to increase in the number of neurons, the total number of neurons in the myenteric plexus was counted in wholemounts from control and inflamed ileum at 7 day post-TNBS injection. The total number of neurons labelled by anti-Hu antibody was 817 ± 110 neurons/10 ganglia in control ileum ($n = 3$) and 676 ± 76 neurons/10 ganglia in inflamed ileum ($n = 3$). Therefore, the loss of about 17% of neurons was observed in the inflamed ileum. The proportion of Dogiel type II neurons relative to the total number of neurons in the ganglia was counted by observing cytoplasmic NeuN staining which occurred on average in 34% of Hu-immunoreactive cells from control ($n = 3$) and 32% from the inflamed ileum ($n = 3$) (Fig. 10).

Therefore, the increased number of processes projecting to the mucosa seen at 7 days post-TNBS treatment was not due to increase in the number of neurons, but most likely was due to neuronal sprouting following the axonal damage that occurred at the early stages.

Discussion

In the present study we found that myenteric Dogiel type II neurons become significantly hyperexcitable and change their electrophysiological properties as early as 3 h after induction of inflammation in the ileum or after sham operation. These changes were associated with damage to the mucosa and its innervation, at times when there is also

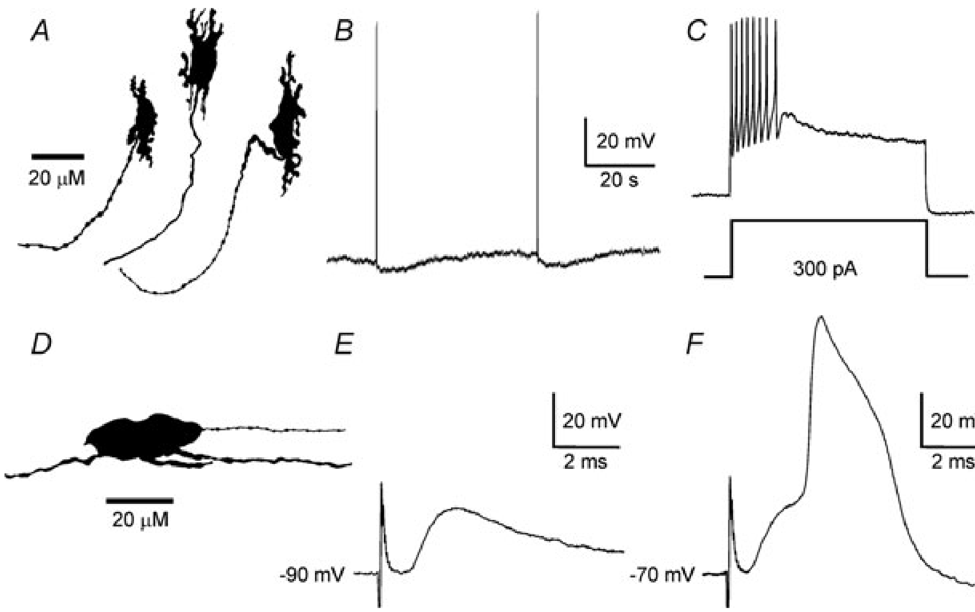

Figure 5. The occurrence of the late afterhyperpolarization (AHP) in myenteric neurons with Dogiel type I (DI) morphology and the fast excitatory postsynaptic potentials (EPSPs) in myenteric Dogiel type II (DII) neurons from the inflamed ileum taken at 24 h after TNBS injection

A, the morphologies of DI neurons from the myenteric plexus that have prominent late AHPs following their single action potentials (*B*) elicited by intracellular current pulses. *C*, a depolarizing current pulse of 300 pA, 500 ms induced a train of action potentials followed by a late AHP. *D*, a DII neuron with prominent fast EPSPs whose amplitudes were enhanced at −90 mV (*E*). *F*, the action potential shape recorded from this DII neuron.

an inflammatory reaction in myenteric ganglia (Linden *et al.* 2005). Functional changes persisted at 24 h in TNBS treated animals, but not in sham. At 7 days after induction of inflammation, re-innervation and sprouting of nerve fibres innervating the mucosa was observed.

Effects of acute inflammation on electrophysiological properties of myenteric Dogiel type II neurons

The present study shows significant neuronal hyperexcitability at early stages (3–24 h) after induction of inflammation in the ileum, defined by very low thresholds for induction of action potentials and greater numbers of action potentials in response to depolarization. Infiltration of immune cells to the areas adjacent to the myenteric plexus have been detected at 2 h after the induction of inflammation by TNBS in the colon (Linden *et al.* 2005).

Similar changes in neuronal excitability occurred in sham operated animals at 3 h after the surgery (discussed below) suggesting that at this time point both TNBS and tissue handling during the surgery contribute to the inflammatory response and changes in electrophysiological properties of neurons. By 24 h, electrophysiological properties of neurons from sham operated animals were not statistically different from the control group. In contrast, in the 24 h TNBS group, neuronal hyperexcitability was at the same level as at 3 h. A previous study showed that hyperexcitability persisted at day 7 after the induction of inflammation in the ileum (Nurgali *et al.* 2007). These changes suggest that the threshold for activation of voltage-sensitive Na^+ currents is significantly reduced in Dogiel type II neurons after inflammation (Nurgali *et al.* 2007). The findings are consistent with previous studies of spinal primary afferent

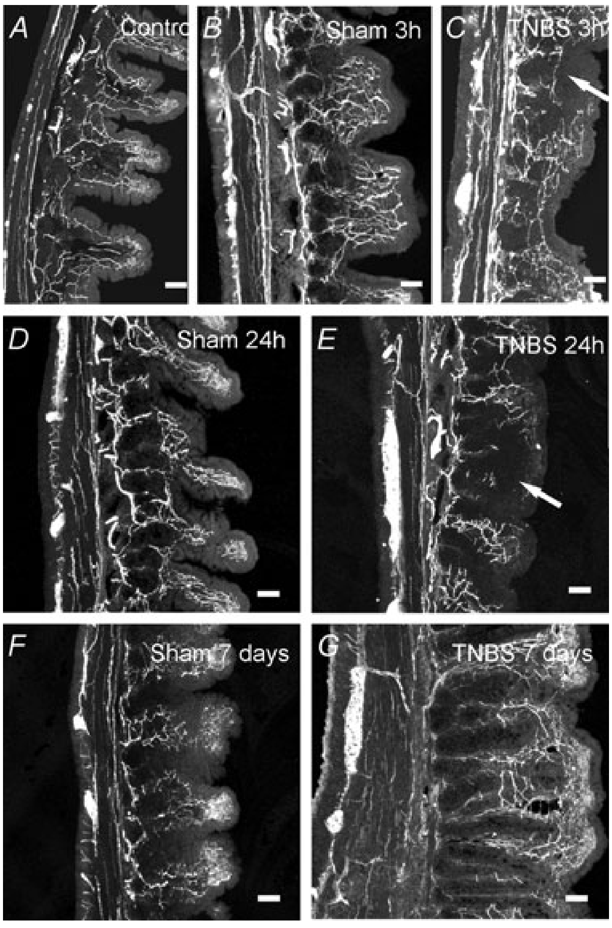

Figure 6. Innervation of the mucosa in cross sections from the control, sham operated and TNBS guinea-pigs taken at 3, 24 h and 7 days post surgery
Nerve bundles innervating villi were revealed by anti-β-tubulin III antibody, which is specific for neuronal tissue. *A*, control ileum. *B*, sham operated ileum taken 3 h after the surgery. *C*, cross section from the inflamed ileum taken 3 h after the TNBS injection. *D*, sham operated ileum taken 24 h after the surgery. *E*, section from the inflamed ileum taken 24 h after the TNBS injection. *F*, sham operated ileum 7 days after the surgery. *G*, inflamed ileum 7 days after the TNBS injection.
Scale bars 50 μm for all images.

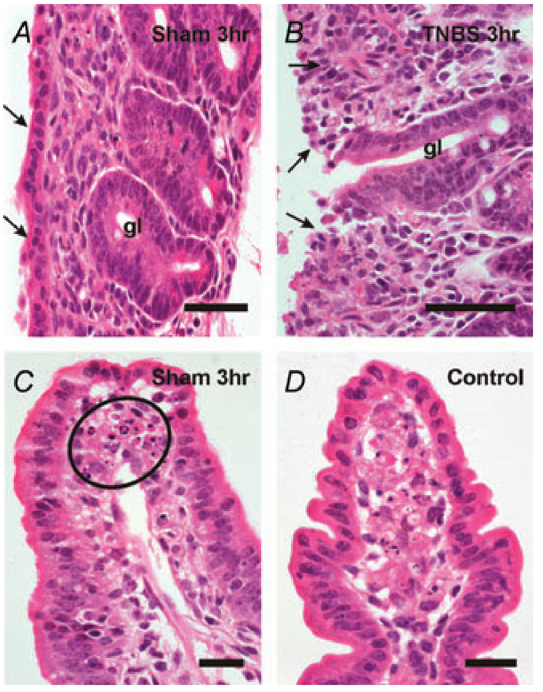

Figure 7. Histological effects observed in sham animals at 3 h. No changes were observed at later times
A, Sham. Region of the surface mucosa which was flattened. The epithelium was intact (arrows). *B*, TNBS. The villi were lost and the epithelium was missing from the exposed surface (arrows). The epithelium of the mucosal glands (gl) was intact in both the TNBS treated (*B*) and in the sham (*A*). *C*, Sham. There was a low-level inflammatory reaction in which an accumulation of inflammatory cells occurred at the villus tip (circled). Note the intact surface epithelium. *D*, control ileum. The accumulation of inflammatory cells observed in the sham is not seen. Scale bars: *A* and *B* = 50 μm; *C* and *D* = 20 μm.

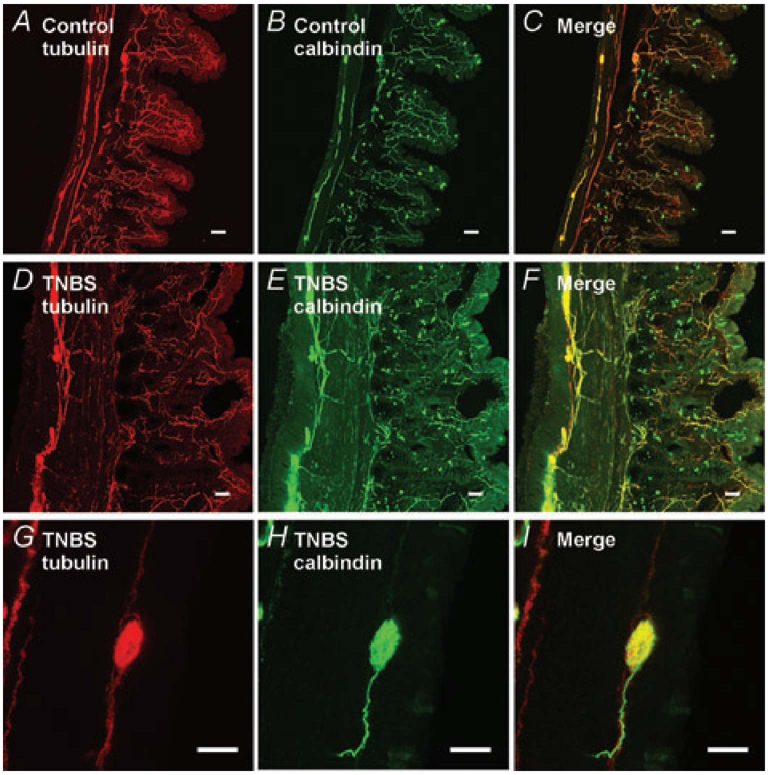

Figure 8. Double staining with anti-β-tubulin III and anti-calbindin antibodies in cross sections from control and 7 day TNBS ileum
β-Tubulin III-immunoreactive processes innervating the mucosa contain calbindin-immunoreactive nerve fibres in the cross sections from control (*A*, *B*, *C*) and inflamed (*D*, *E*, *F*) ileum. Scale bars 50 μm. The cell body of the Dogiel type II neuron in the myenteric plexus labelled by anti-β-tubulin (*G*) and anti-calbindin (*H*) antibodies and merged (*I*). Scale bars 20 μm.

neurons that demonstrated potentiation of Na^+ currents in response to inflammation or inflammatory mediators (Stewart *et al.* 2003; Beyak *et al.* 2004; Beyak & Vanner, 2005; Maingret *et al.* 2008). Extrinsic, dorsal root ganglion (DRG), primary afferent neurons, identified by retrograde tracing to project to the mucosa, also become hyperexcitable after induction of colitis, and a lack of effect in non-labelled neurons suggests that signalling originated at the nerve terminals rather than through circulating mediators acting at the cell bodies in the DRG (Stewart *et al.* 2003; Beyak *et al.* 2004). Findings in myenteric and submucosal neurons from the guinea-pig colon also demonstrated an increase in excitability of Dogiel type II neurons (Linden *et al.* 2003; Lomax *et al.* 2005). The bursts of anodal break firing induced by small hyperpolarizing current pulses at 3 and 24 h post-TNBS injection (Fig. 1*Bc* and *Cc*) are also a signature of neuronal hyperexcitability.

Our results demonstrated an increase in the duration of the action potentials without change in the action potential amplitude at 3 and 24 h after TNBS injection. Similarly, an

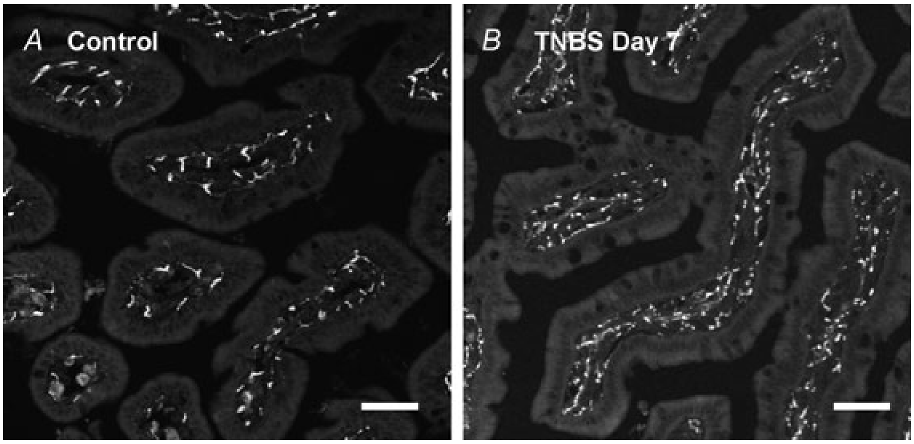

Figure 9. Increase in the number of nerve bundles projecting to the mucosa observed at day 7 after the injection of TNBS, compared to the control ileum
Nerve bundles innervating the villi were revealed by anti-β-tubulin III antibody in transverse sections taken at the middle level of villi in tissues from control (*A*) and inflamed (*B*) ileum. Scale bars 50 μm.

increase in the action potential duration was observed in submucosal neurons after TNBS-induced inflammation in the guinea-pig ileum (Hons *et al.* 2009). This might be due to enhancement of N- and/or R-type voltage-gated Ca^{2+} currents, which are prominent in Dogiel type II neurons (Rugiero *et al.* 2002; Bian *et al.* 2004; Needham *et al.* 2010) or inhibition of delayed rectifier K^+ current which underlies the repolarization phase of the action potential (Zholos *et al.* 1999; Ren *et al.* 2000). These findings reveal that the currents underlying the generation of action potentials are significantly affected by acute inflammation, but further studies have to be done to investigate the role of these channels in the change of the action potential shape following induction of inflammation in the ileum.

The amplitude of the late AHP following the action potential was significantly reduced at 3 and 24 h after TNBS injection. Inhibition of the late AHP, which contributes to the hyperexcitability of Dogiel type II neurons, has been previously reported in TNBS-induced colitis, 6–7 days after induction of inflammation (Linden *et al.* 2003; Lomax *et al.* 2005). The channels underlying the late AHP current (I_{AHP}) in myenteric Dogiel type II neurons are Ca^{2+}-activated intermediate conductance K^+ channels (IK_{Ca}) (Vogalis *et al.* 2002; Neylon *et al.* 2004; Nguyen *et al.* 2007). IK_{Ca} channels are regulated by protein kinases C and A, which can inhibit a late AHP without

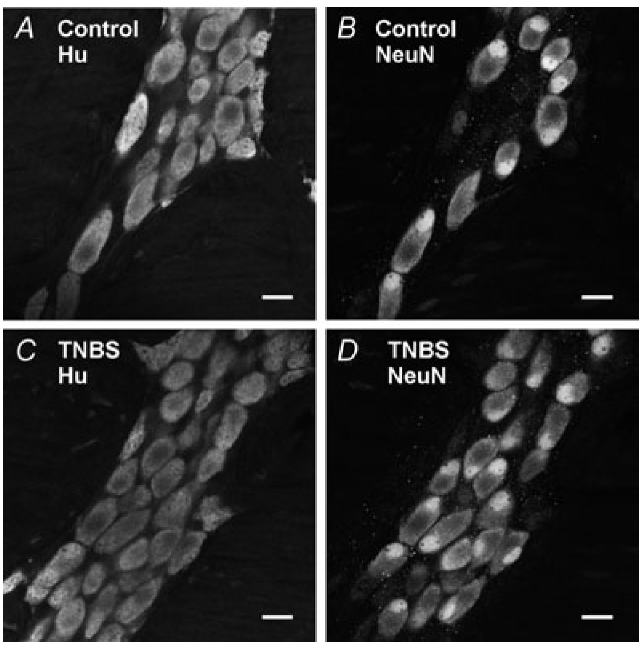

Figure 10. The proportion of Dogiel type II (DII) neurons in the myenteric plexus counted at 7 days after the induction of inflammation
The total number of neurons was labelled by anti-Hu antibody and counted in wholemounts from control (*A*) and inflamed (*C*) ileum. The proportion of DII neurons revealed by cytoplasmic anti-NeuN antibody staining in control (*B*) and inflamed (*D*) ileum was counted relative to the total number of neurons. Scale bars 20 μm.

suppressing the Ca^{2+} component of the action potential (Kawai, 2003; Vogalis *et al.* 2003). It has been shown that inflammatory mediators can activate protein kinases A and C, which in turn affect ion currents and augment neuronal firing without necessarily changing membrane potential (Gold *et al.* 1998; Devor, 2006). Similar to previous reports at day 6–7 after TNBS injection (Linden *et al.* 2003; Lomax *et al.* 2005; Nurgali *et al.* 2007) no changes in the resting membrane potential were observed in Dogiel type II neurons at 3 and 24 h after TNBS injection in the present study.

Axonal damage and sprouting following mucosal damage and inflammation

In the present study we have demonstrated that significant axonal damage occurs at 3–24 h after TNBS-induced inflammation in the guinea-pig ileum. It should be noted that all nerve fibres projecting to mucosa are exposed to an inflammatory environment. Prominent amongst the nerve fibres innervating the mucosa are the terminals of myenteric Dogiel type II neurons (Furness *et al.* 1990; Song *et al.* 1991, 1994), and the projections of submucosal neurons, including both DII neurons and secretomotor neurons (Furness, 2006). According to Song *et al.* (1991, 1994), all myenteric DII neurons, most of which are calbindin immunoreactive, project to the mucosa and each villus is estimated to be supplied by axons of at least five Dogiel type II neurons. The present study showed that the majority of β-tubulin immunoreactive fibre bundles projecting to the mucosa also contained calbindin-positive fibres. The majority of calbindin-immunoreactive fibres innervating the mucosa have intrinsic origin from the Dogiel type II neurons; only a minority of calbindin-positive fibres belong to extrinsic afferent neurons innervating the gut (Furness *et al.* 1990; Kuramoto *et al.* 1990). Therefore, nerve fibres projecting to the mucosa that were affected by inflammation included processes of Dogiel type II neurons from the myenteric plexus.

Although the number of Hu-immunoreactive nerve cells decreased, the number of nerve fibre bundles innervating the mucosa increased at 7 days post-TNBS injection, compared to control. A reduction in the number of neurons in the myenteric plexus occurs within the first 12 h after induction of inflammation by TNBS in the guinea-pig colon (Linden *et al.* 2005) and between 0.5 and 3 h in a model of dinitrobenzene sulfonate (DNBS)-induced colitis in mice (Boyer *et al.* 2005). The total number of neurons remained at the decreased level long after the acute stage of inflammation in TNBS-induced colitis in the guinea-pigs (Linden *et al.* 2005), rats (Lin *et al.* 2005) and DNBS-induced colitis in rats (Sanovic *et al.* 1999). In our study the total number of myenteric neurons revealed by anti-Hu antibody in the guinea-pig ileum was reduced by about 17%. This is consistent with previously reported loss of about 15% of myenteric neurons in the guinea-pig distal colon after TNBS injection (Linden *et al.* 2005). Similar to the Linden *et al*. (2005) data, we found that the proportion of myenteric Dogiel type II neurons in the ileum labelled by cytoplasmic NeuN has not changed suggesting that the total number of these neurons and of other types of neurons are reduced. Despite the loss of Dogiel type II neurons, there is an increase in fibre density in the mucosa at 7 days post-TNBS injection. These data imply that re-innervation of villi and axonal sprouting by the smaller number of remaining neurons occurs.

Similar to our findings, initial degeneration during the acute phase of inflammation and a subsequent re-innervation and sprouting of nerve fibres innervating the mucosa has been reported in the jejunum of rats infected by *Nippostrongylus brasiliensis* (Stead *et al.* 1991). It has been reported that axon numbers are reduced in the smooth muscle during the acute stage of inflammation followed by a high degree of axonal proliferation of both intrinsic and extrinsic fibres in severe TNBS-induced transmural inflammation of the rat colon (Lourenssen *et al.* 2005). Axonal damage has also been described in samples of small intestine from patients with Crohn's disease (Dvorak *et al.* 1980). The distribution of axons containing calcitonin gene-related peptide, substance P and vasoactive intestinal peptide showed extensive changes during TNBS-induced colitis in rats, with an initial reduction of the number and density of nerve fibres in the mucosa followed by increased expression and a return to control values on day 7 after TNBS injection (Miampamba & Sharkey, 1998). These studies and our results suggest that axonal proliferation occurs in the inflamed small and large intestine.

Exposure of nerve terminals in the mucosa or of nerve cells to inflammatory mediators and neurotoxic factors produced by immune cells and damaged tissues appears to be an essential factor contributing to the neuronal hyperexcitability. The lack of hyperexcitability in neurons from longitudinal muscle-myenteric plexus preparations from control tissues, where axons projecting to mucosa have been severed in our experiments, suggests that merely severing the axons does not lead to induction of neuronal excitability. Consistent with an essential role of inflammatory mediators, treatment with a COX-2 inhibitor on days 2–6 after TNBS injection reduced hyperexcitability of myenteric Dogiel type II neurons in the guinea-pig colon, at 6 days post-TNBS, to control level, without reducing mucosal damage (Linden *et al.* 2004).

The hyperexcitability of Dogiel type II neurons persists for at least 2 months after the resolution of acute inflammation (Krauter *et al.* 2007*b*; Lomax *et al.* 2007). Thus, the mechanisms that initiate neuronal hyperexcitability at the acute stage of inflammation and mechanisms that maintain changes in the absence of active inflammation of enteric ganglia might be different. For example, it has been suggested that neuronal hyperexcitability at later stages after the resolution of inflammation might be due to a persistent alteration in channel expression and/or a continuous release of inflammatory mediators due to a low grade inflammation (Lomax *et al.* 2007).

Changes in electrophysiological properties of Dogiel type II neurons in sham operated guinea-pigs

Some Dogiel type II neurons at 3 h after sham operation had significant changes in their electrophysiological properties, similar to the neurons from the inflamed ileum. These changes correlated with histological and immunohistochemical evidence of morphological change in the mucosa that occurred at 3 h after sham operations. Both the morphological and the electrophysiological changes were resolved and returned to control levels by 24 h after the sham operations. It has been shown that an intestinal insult such as intraoperative handling of the bowel results in a significant inflammatory response after the surgery (Kalff *et al.* 1998; Schwarz *et al.* 2004). This response is characterized by infiltration of monocytes and neutrophils and activation of the resident macrophages, which then release cytokines, chemokines, and other inflammatory mediators (e.g. nitric oxide and prostaglandins) (Kalff *et al.* 1998, 1999, 2003). Our previous study has shown that sham operations cause minor inflammation in the ileum with transient elevation of immune cells (eosinophils and T lymphocytes) adjacent to submucosal ganglia within the first 6 h which subsided close to control level by 24 h (Pontell *et al.* 2009). Therefore, changes in electrophysiological properties of enteric neurons in sham operated animals at 3 h after the surgery can be due to the consequences of tissue handling during sham operation.

Conclusion

Our present study has demonstrated hyperexcitability of myenteric Dogiel type II neurons and damage to the neuronal processes projecting to the mucosa at 3 and 24 h after induction of inflammation. At the same time, there is proliferation of immune cells in myenteric ganglia (Linden *et al.* 2005). A peak of neuronal hyperexcitability (3–24 h) coincides with severe mucosal damage and the peak of inflammatory response in the ileum after TNBS treatment (Pontell *et al.* 2009). Exposure of neurons and their terminals to inflammatory mediators and neurotoxic factors produced by immune cells and damaged tissues appear to be important factors contributing to the neuronal hyperexcitability at the acute stage of inflammation. Re-innervation and sprouting of nerve fibres projecting to mucosa have been observed at 7 days after the resolution of acute inflammation. The mechanisms underlying neuronal hyperexcitability at the acute stage of inflammation might be different from those underlying long-term changes in the absence of active inflammation in the ganglia.

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Author contributions

K.N. designed the research, conducted electrophysiological experiments, analyzed data and wrote the manuscript. Z.Q. performed the immunohistochemistry and analyzed data. B.H. and M.T. carried out animal surgeries and contributed to drafting of the manuscript. L.P. performed histology and animal surgeries. J.B.F. performed animal surgeries and contributed to the manuscript writing. All authors approved the manuscript for publication. Experiments were conducted in the Departments of Physiology and Anatomy & Cell Biology, The University of Melbourne, Australia.

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